

On page 16, line 22, delete "Sequence 2" and insert therefor --SEQ ID NO:2--.

In the claims:

On page 38, line 3, delete "listed in Sequence 1" and insert therefor --according to SEQ ID NO:1--.

On page 38, line 5-6, delete "of Sequence 1" and insert therefor --according to SEQ ID NO:1--.

On page 38, line 8-9, delete "of Sequence 1" and insert therefor --according to SEQ ID NO:1--.

On page 39, line 1, delete "listed in Sequence 2" and insert therefor --according to SEQ ID NO:2--.

On page 39, line 3-4, delete "of Sequence 2" and insert therefor --according to SEQ ID NO:2--.

On page 39, line 6-7, delete "of Sequence 2" and insert therefor --according to SEQ ID NO:2--.

On page 39, line 20, delete "listed in Sequence 3" and insert therefor --according to SEQ ID NO:3--.

On page 40, line 1-2, delete "of Sequence 3" and insert therefor --according to SEQ ID NO:3--.

REMARKS

The Notice to File Corrected Application Papers indicated that an abstract was not provided with the application as required under 37 CFR 1.72(b). An abstract was printed on the cover page of PCT/KR00/00173. A copy of the abstract is filed herewith as a separate page.

The Notice also indicates that the Application fails to comply with the requirements of 37 C.F.R. 1.8211-1.825. The application has been amended to contain reference to the Sequence Listing. A request under 37 C.F.R. 1.821(e) to incorporate the computer readable copy of the Sequence Listing and a statement that the paper copy enclosed herewith is identical to the sequence listing in 09/674,615 is enclosed.

CONCLUSIONS

The application is believed to be in condition for substantive examination on the merits of the case. A prompt and positive response is earnestly requested.

Respectfully submitted,
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APPENDIX
CLEAN COPY OF THE CLAIMS

1. A *PRCI* gene derived from *Hansenula polymorpha* DL1 (ATCC 26012), having the base sequence according to SEQ ID NO: 1, which codes for carboxypeptidase Y.

2. A vector pHYL, which is constructed by inserting a *Hansenula polymorpha* *LEU2* gene into a plasmid pHDY2 (KCTC 0732BP) carrying the *PRCI* gene according to SEQ ID NO: 1 in such a way that the *PRCI* gene is disrupted.

3. A vector pHYUZ, which is constructed by inserting a *Hansenula polymorpha* *URA3* gene pop-out cassette into a plasmid pHDY2 carrying the *PRCI* gene according to SEQ ID NO: 1 in such a way that the *PRCI* gene is disrupted.

4. A carboxypeptidase Y mutant strain, into which a *Hansenula polymorpha* UR2 strain is transformed with the vector pHYL of claim 2.

5. A carboxypeptidase Y mutant strain, *Hansenula polymorpha* DL1/ *cpy*(KCTC 0735 BP), into which a *Hansenula polymorpha* DL1 strain is transformed with the vector pHYUZ of claim 3.

6. A process for producing a recombinant protein, wherein a gene coding for an exogenous protein is introduced into the carboxypeptidase Y mutant strain of claim 4 and the cell is cultured in a culture medium.

7. A process for producing a recombinant protein, wherein a gene coding for an exogenous protein is introduced into the carboxypeptidase Y mutant strain of claim 5 and the cell is cultured in a culture medium.

8. A recombinant protein, which is produced by the process of claim 6 or 7.

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9. A *PEP4* gene derived from *Hansenula polymorpha* DL1 (ATCC 26012), having the base sequence according to SEQ ID NO: 2, which codes for protease A.

10. A vector pHPL, which is constructed by inserting a *Hansenula polymorpha* *LEU2* gene into a plasmid pHDP4 (KCTC 0733BP) carrying the *PEP4* gene according to SEQ ID NO: 2 in such a way that the *PEP4* gene is disrupted.

11. A vector pHPUZ, which is constructed by inserting a *Hansenula polymorpha* *URA3* gene pop-out cassette into a plasmid pHDP4 carrying the *PEP4* gene according to SEQ ID NO: 2 in such a way that the *PEP4* gene is disrupted.

12. A protease A mutant strain, into which a *Hansenula polymorpha* UR2 strain is transformed with the vector pHPL of claim 10.

13. A protease A mutant strain, *Hansenula polymorpha* DL1/ *pep4*(KCTC 0734 BP), into which a *Hansenula polymorpha* DL1 strain is transformed with the vector pHPUZ of claim 11.

14. A process for producing a recombinant protein, wherein a gene coding for an exogenous protein is introduced into the protease A mutant strain of claim 12 and the cell is cultured in a culture medium.

15. A process for producing a recombinant protein, wherein a gene coding for an exogenous protein is introduced into the protease A mutant strain of claim 13 and the cell is cultured in a culture medium.

16. A recombinant protein, which is produced by the process of claim 14 or 16.

17. A *KEX1* gene derived from *Hansenula polymorpha* DL1 (ATCC 26012), having the base sequence according to SEQ ID NO: 3, which codes for carboxypeptidase α .

18. A vector pKUZ, which is constructed by inserting a *Hansenula polymorpha* *URA3* gene pop-out cassette into a plasmid pKH3.9 carrying the *KEX1* gene according to SEQ ID NO: 3 in such a way that the *KEX1* gene is disrupted.

19. A carboxypeptidase α mutant strain, *Hansenula polymorpha* DL1/ *kex1*(KCTC 0736 BP), into which a *Hansenula polymorpha* DL1 strain is transformed with the vector pKUZ of claim 18.

20. A process for producing a recombinant protein, wherein a gene coding for an exogenous protein is introduced into the carboxypeptidase α mutant strain of claim 19 and the cell is cultured in a culture medium.

21. A recombinant protein, which is produced by the process of claim 20.

22. A carboxypeptidase Y/protease A mutant strain, into which a *Hansenula polymorpha* DL1 strain is transformed with the vector pHYUZ of claim 3 and the vector pHPUZ of claim 11.

23. A process for producing a recombinant protein, wherein a gene coding for an exogenous protein is introduced into the carboxypeptidase Y/protease A mutant strain of claim 22 and the cell is cultured in a culture medium.

24. A recombinant protein, which is produced by the process of claim 23.

25. A carboxypeptidase Y/carboxypeptidase α mutant strain, into which a *Hansenula polymorpha* DL1 strain is transformed with the vector pHYUZ of claim 3 and the vector pKUZ of claim 20.

26. A process for producing a recombinant protein, wherein a gene coding for an exogenous protein is introduced into the carboxypeptidase Y/carboxypeptidase α mutant strain of claim 25 and the cell is cultured in a culture medium.

27. A recombinant protein, which is produced by the process of claim 26.

28. A recombinant protein in accordance with claim 27, wherein the recombinant protein is human epidermal growth factor (hEGF).

29. A protease A/carboxypeptidase α mutant strain, into which a *Hansenula polymorpha* DL1 strain is transformed with the vector pHPUZ of claim 11 and the vector pKUZ of claim 18.

30. A process for producing a recombinant protein, wherein a gene coding for an exogenous protein is introduced into the protease A/carboxypeptidase α mutant strain of claim 29 and the cell is cultured in a culture medium.

31. A recombinant protein, which is produced by the process of claim 30.

32. A carboxypeptidase Y/protease A/carboxypeptidase α mutant strain, into which a *Hansenula polymorpha* DL1 strain is transformed with the vector pHYUZ of claim 3, the vector pHPUZ of claim 11, and the vector pKUZ of claim 18.

33. A process for producing a recombinant protein, wherein a gene coding for an exogenous protein is introduced into the carboxypeptidase Y/protease A/carboxypeptidase α mutant strain of claim 32 and the cell is cultured in a culture medium.

34. A recombinant protein, which is produced by the process of claim 33.

35. A vector pMLT-delta (KCTC 0727BP), which carries as a selective marker for *Hansenula polymorpha* a *Saccharomyces cerevisiae* *LEU2* inserted between a portion of a *MOX* promoter and a DNA fragment comprising a portion of a *TRP3* gene (mox(p)::*S.cerevisiae* *LEU*::*trp3*).

36. A *Hansenula polymorpha* *mox* mutant strain, which is prepared by introducing the vector pMLT-delta of claim 35 into *Hansenula polymorpha* in such a way that the *MOX-TRP3* gene of *Hansenula polymorpha* is disrupted.

37. A *Hansenula polymorpha* *mox* mutant strain in accordance with claim 36, wherein the *Hansenula polymorpha* is a *Hansenula polymorpha* DL1 strain.

38. A *Hansenula polymorpha* *mox* mutant strain DLT2 (KCTC 0728BP), which is prepared by introducing the vector pMLT-delta of claim 35 into *Hansenula polymorpha* DL1-L in such a way that the *MOX-TRP3* gene of *Hansenula polymorpha* is disrupted.

39. A process for producing a recombinant protein, wherein the *Hansenula polymorpha* *mox* mutant strain of any of claims 36 to 38 is transformed with an expression cassette and cultured in a methanol medium, serving as a recombinant protein expression host, said expression cassette comprising a promoter whose expression is induced by methanol.

40. A process in accordance with claim 39, wherein the *Hansenula polymorpha* *mox* mutant strain is first cultured to a high concentration in a medium containing glycerol as a carbon source and then, cultured in a medium containing methanol as a carbon source.

41. A recombinant protein, which is produced by the process of claim 39 or 40.

42. A process for popping-out an expression cassette integrated into the *MOX* gene site of the *mox* strain DLT2.

43. A process for developing novel mutant strains derived from a recombinant DLT2 strain, which is prepared in accordance with claim 38, into a host for general use in producing various recombinant proteins using the pop-out technique of claim 42.

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page 9, lines 22-29

A *Sacchromyces cerevisiae* carboxypeptidase Y gene (*PRC1*) is amplified by PCR. This PCR product is used as a probe in detecting a corresponding *Hansenula polymorpha* gene (*PRC1*) through Southern blotting. First, the genome of *Hansenula polymorpha* DL-1 is treated with restriction enzymes and hybridized with the probe. A DNA band is detected from a 3 kb *Pst*I DNA fragment which is, then, inserted into a plasmid for preparing a DNA library. After extensive Southern blotting processes, a plasmid carrying a *Hansenula polymorpha PRC1* gene was selected. After the DNA fragment is reduced into a 2.2 kb *Xho*I/*Pst*I fragment which is then used to construct plasmid pHY2. The base sequence of the gene is read as given in SEQ ID NO:1.

page 10, lines 1-13

Well-known amino acid sequences of carboxypeptidase α of many strains are analyzed to select high homology regions. On the basis of the amino acid sequences of the high homology regions, primers are designed. A PCR using the primers resulted in the amplification of a *KEX1* DNA fragment 306 bp long, from the *Hansenula polymorpha* DL-1 genome. This PCR product is used as a probe in detecting a whole *Hansenula polymorpha* gene *KEX1* through Southern blotting. First, the genome of *Hansenula polymorpha* DL-1 is treated with restriction enzymes and hybridized with the probe. A DNA band is detected from a 4.5 kb *Hind*III DNA fragment which is, then, inserted into a plasmid for preparing a DNA library. After extensive Southern blotting processes, a plasmid carrying a *Hansenula polymorpha KEX1* gene was selected. After the DNA fragment is reduced into a 3.9 kb *Eco*RI/*Hind*III fragment which is then used to construct plasmid pKH3.9. The base sequence of the gene is read as given in SEQ ID NO: 3.

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page 12, line 7 to page 13, line 1-29

First step: Construction of Probes for Cloning *Hansenula polymorpha* Genes *PRC1*, *PEP4* and *KEX1*

In order to obtain a *Saccharomyces cerevisiae* *PRC1* gene, there were used the following primers:

Primer C1(SEQ ID NO:4): 5 -ATG AAA GCA TTC ACC AG-3

Primer C2(SEQ ID NO:5): 5 -TTA TAA GGA GAA ACC AC-3

With the aid of the primers C1 and C2, 25 cycles of PCR, each consisting of a denaturing step at 94 °C for 30 sec, an annealing step at 55 °C for 30 sec and an extending step at 72 °C for 2 min, resulted in the acquisition of a *Sacchromyces cerevisiae* *PRC1* gene 1.6 kb long while the genomic DNA of *Sacchromyces cerevisiae* served as a template for the enzyme. Using a DIG-labelling and detection kit, commercially available from Boehringer Mannheim, the PCR product was labeled according to the indications of the kit manual to give a probe for cloning a *PRC1* gene of *Hansenula polymorpha*.

Synthesized for the amplification of a *Sacchromyces cerevisiae* *PEP4* gene were the following primers:

Primer P1(SEQ ID NO:6): 5 -ATG TTC AGC TTG AAA GC-3

Primer P2(SEQ ID NO:7): 5 -TCA AAT TCG TTT GGC C-3

The *Sacchromyces cerevisiae* *PEP4* gene 1.22 kb long was obtained from the *Sacchromyces cerevisiae* genomic DNA through 25 cycles of PCR, each consisting of a denaturing step at 94 °C for 30 sec, an annealing step at 55 °C for 30 sec and an extending step at 72 °C for 2 min. This *PEP4* gene was labeled in the same manner as in the above *Hansenula polymorpha* *PRC1* gene, so as to give a probe for cloning a *PEP4* gene of *Hansenula polymorpha*.

page 13, lines 2-12

As for a probe for detecting a *Hansenula polymorpha KEX1* gene, it was obtained by PCR using the following primers:

Primer K1(SEQ ID NO:8): 5'-TGG YTS AAC GGH CCW GGH TGY TCB
TCB-3

Primer K2(SEQ ID NO:9): 5'-WGG RAT GTA YTG WCC RGC GTA VGA
CTC DCC-3

In this regard, five cycles of a PCR, each consisting of a denaturing step at 94 °C for 30 sec, an annealing step at 50 °C for 30 sec and an extending step at 72 °C for 30 sec was conducted, followed by performing 20 cycles of a PCR under the heat condition consisting of a denaturing step at 94 °C for 30 sec, an annealing step at 55 °C for 30 sec and an extending step at 72 °C for 30 min to amplify a *Hansenula polymorpha KEX1* DNA segment 306 bp. This DNA segment was labeled in the same manner as in the above to prepare a probe for cloning a *Hansenula polymorpha PRC1* gene.

page 13, lines 3-23

Next, the DNA fragment was isolated from the position at which the blue band appeared, and ligated to plasmid pBluescript KSII⁺ with which *E. coli* DH5 was transformed to prepare a DNA library. This DNA library was subjected repetitively to Southern blotting to select a plasmid carrying the *PRC1* gene, called plasmid pHDY1. Double digestion with restriction enzymes *XhoI/PstI* reduced the DNA fragment from about 3 kb to about 2.2 kb. The plasmid harboring the *XhoI/PstI* DNA fragment, was called pHDY2. It was deposited in the Korean Collection for Type Culture (KCTC), placed in Korea Research Institute of Bioscience and Biotechnology (KRIBB), #52, Oun-dong, Yusong-ku, Taejon 305-333, Republic of Korea, on the date of Feb. 18, 2000 and it was accepted under the accession number of KCTC 0732BP. The restriction site mapping and base sequencing of the *Hansenula polymorpha* DL1 *PRC1* gene was conducted as illustrated in Fig. 1. The base sequence of the *PRC1* gene is given in SEQ ID NO:1. This DNA sequence was registered as U67174 with GenBank on Aug. 17, 1996. Analysis of the base sequence

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page 14, line 25 to page 25, line 24

In order to obtain a *PEP4* gene from the genomic DNA of *Hansenula polymorpha*, Southern blotting was conducted with the probe prepared in the first step. First, after six aliquots of the genomic DNA obtained in the second step were treated with restriction enzymes *Bam*HI, *Eco*RI, *Eco*RV, *Hind*III, *Pst*I, and *Sal*I, respectively, the resulting DNA fragments were fractionated on a 0.9% agarose gel by electrophoresis. The separated DNA molecules were transferred to a Nytran membrane (Schleicher & Schuell) by blotting, followed by exposing the membrane to the labeled probe under conditions favoring hybridization. The hybridization was conducted in the same manner as in the third step. A band that was dyed blue was observed in a DNA fragment which was cut into a length of about 8 kb by restriction enzyme *Bam*HI. Next, the DNA fragment was isolated from the position at which the blue band appeared, and used to prepare a DNA library in the same manner as in the third step. The DNA library was subjected repetitively to Southern blotting to select a plasmid carrying the *PRC1* gene, called plasmid pHDP3. Double digestion with restriction enzymes *Sac*I/*Hind*III reduced the DNA fragment from about 8 kb to about 2.0 kb. The plasmid harboring the *Sac*I/*Hind*III DNA fragment, was called pHDY4. It was deposited in the Korean Collection for Type Culture (KCTC), placed in Korea Research Institute of Bioscience and Biotechnology (KRIBB), #52, Oun-dong, Yusong-ku, Taejon 305-333, Republic of Korea, on the date of Feb. 18, 2000 and it was accepted under the accession number of KCTC 0733BP. The restriction site mapping and base sequencing of the *Hansenula polymorpha* DL1 *PEP4* gene was conducted as illustrated in Fig. 2. The base sequence of the *PEP4* gene is given in SEQ ID NO:2. This DNA sequence was registered as U67173 with GenBank on Aug. 17, 1996. Analysis of the base sequence revealed that the *Hansenula polymorpha* DL1 *PEP4* gene is 1,242 bp long with no introns and shows 52.4% homology to the base sequence of a *Saccharomyces cerevisiae* *PRC1* gene. When being deduced from SEQ ID NO:2, the amino acid sequence of the *Hansenula polymorpha* DL1 *PEP4* gene exhibits 50% homology to the protease A of *Saccharomyces cerevisiae*. In addition, high homology can be found in the 117th amino acid residue, which is identified to be an aspartic acid acting as a catalytic group within an active site of aspartyl protease.

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page 16, lines 8-29

Next, the DNA fragment was isolated from the position at which the blue band appeared, and used to prepare a DNA library as in the third step. The DNA library was subjected repetitively to Southern blotting to select a plasmid carrying the *PRC1* gene, called plasmid pKH4.5. The plasmid pKH4.5 was deposited in the Korean Collection for Type Culture (KCTC), placed in Korea Research Institute of Bioscience and Biotechnology(KRIBB), #52, Oun-dong, Yusong-ku, Taejon 305-333, Republic of Korea, on the date of Feb. 18, 2000 and it was accepted under the accession number of KCTC 0731BP. Double digestion with restriction enzymes *EcoRI/HindIII* reduced the DNA fragment from about 4.5 kb to about 3.9 kb. The plasmid harboring the *EcoRI/HindIII* DNA fragment, was called pKH3.9. The restriction site mapping and base sequencing of the *Hansenula polymorpha* DL1 *PRC1* gene was conducted as illustrated in Fig. 3. The base sequence of the *PRC1* gene is given in SEQ ID NO:3. This DNA sequence was registered as AF090325 with GenBank on Sep. 4, 1998. Analysis of the base sequence revealed that the *Hansenula polymorpha* DL1 *KEX1* gene is 1,833 bp long with no introns. When being deduced from SEQ ID NO:2, the amino acid sequence of the *Hansenula polymorpha* DL1 *KEX1* gene exhibits as low as 20% homology to the carboxypeptidase α of *Saccharomyces cerevisiae*. However, in the 176th amino acid residue, which is identified to be a serine acting as a catalytic group within an active site of serine protease, there is found high homology to carboxypeptidase α as well as carboxypeptidase Y. Amino acid analysis according to Von Heijne's method (Von Heijne, *J. Mol. Biol.*, 173: 243 (1984)) divulged the presence of a signal peptide consisting of 18 amino acid residues.